DNA methylation constitutes an important epigenetic factor in the control of genetic information. In this study, we analyzed expression of the DNA methyltransferase gene and examined DNA methylation patterns during early development of the zebrafish. Maternal transcripts of the zebrafish DNA methyltransferase gene (MTase) are ubiquitously present at high levels in early embryos with overall levels decreasing after the blastula stage. At 24 h, methyltransferase mRNA is predominantly found in the brain, neural tube, eyes, and differentiating somites. Expression of MTase in the somites is highest in the anterior cells of the somites. Despite the high levels of MTase mRNA in blastula-stage embryos, we observe DNA hypomethylation at the blastula and gastrula stages compared to sperm or older embryos. Zebrafish embryos treated with 5-azacytidine (5-azaC) and 5-aza-2-deoxycytidine (5-azadC), nucleotide analogs known to induce cellular differentiation and DNA hypomethylation in mammalian cells, exhibit DNA hypomethylation and developmental perturbations. These defects are specifically observed in embryos treated at the beginning of the blastula period, just prior to midblastula transition. The most common phenotype is the loss of tail and abnormal patterning of somites. Head development is also affected in some embryos. Histological and in situ hybridization analyses reveal whole or partial loss of a differentiated notochord and midline muscle in treated embryos. When examined during gastrulation, 5-azaC-treated embryos have a shortened and thickened axial mesoderm. We propose that DNA methylation is required for normal gastrulation and subsequent patterning of the dorsal mesoderm.© 1999 Academic Press

Key Words: 5-azacytidine; methylation; DNA methyltransferase; notochord; somites; zebrafish.

INTRODUCTION

The generation of a multicellular organism from a fertilized egg requires the establishment of multiple cell lineages with distinct properties. In vertebrates, the establishment of diverse cell lineages, each committed to a defined differentiation pathway, is believed to be dependent upon epigenetic processes that limit the arrays of genes that are expressed, and thus define the cellular phenotype. The difficulty in generating viable progeny after transplantation of nuclei from fetal and adult somatic cells into enucleated oocytes (King and Briggs, 1956; Gurdon et al., 1975; McGrath and Solter, 1984; Di Berardino, 1987), despite recent successes (Wilmut et al., 1997; Wakayama et al., 1998), supports this view of epigenetic control of development. To understand the mechanisms that underlie normal embryogenesis, it is necessary to understand the mechanisms by which such epigenetic control of gene expression is achieved.

DNA methylation offers an attractive mechanism that may account for much of the epigenetic control of gene regulation that occurs during normal embryogenesis. The most prevalent form of DNA methylation in vertebrates is methylation of cytosine residues present as CpG dinucleotides, a modification that can be established and maintained by cytosine-5-DNA methyltransferase (MTase) (Bestor and Verdine, 1994). DNA methylation affects the ability of transcriptional regulatory proteins to bind to DNA (Ben-Hattar et al., 1989), including repressor proteins like MeCP1 and MeCP2 (methyl-cytosine binding proteins) (Boyes and Bird, 1991, 1992), or nucleosome positioning.
(Englander et al., 1993). Increases in DNA methylation are associated with many cases of gene silencing, and reductions in DNA methylation are often associated with gene activation (Cedar, 1988; Michalowsky and Jones, 1989). DNA methylation can be preserved through the replication process by the action of maintenance methylases, which use the replicated hemimethylated DNA as a template (Gruenbaum et al., 1982). DNA methylation can be removed by passive demethylation if methylation after replication is suppressed, and possibly by active demethylation (Weiss and Cedar, 1997). As a result, DNA methylation is clonally heritable, stable, and reversible, and thus satisfies the major requirements for an epigenetic mechanism that can control gene expression.

If DNA methylation contributes to the epigenetic control of development, then it is expected that DNA methyltransferase is expressed in the developing embryo, and that the expression or activity of DNA methyltransferase must exhibit stage and tissue specificity. It is also expected that disruptions in the normal progression of developmentally regulated changes in DNA methylation should result in developmental abnormalities.

The nucleotide analogs 5-azacytidine (5-azaC) and 5-aza-2-deoxycytidine (5-azadC) are routinely used to interfere with DNA methylation in a number of mammalian cell culture systems (reviewed in Cedar and Razin, 1990). These analogs inhibit methylation by incorporation into DNA (Jones and Taylor, 1980) or by interfering with the action of the enzyme cytosine-5–DNA methyltransferase (Santi et al., 1983). Treatment of cells with 5-azaC often results in cell differentiation (transformation) and activation of silent genes (Grant and Worton, 1989). Exposure of embryos to 5-azaC and related compounds produces developmental abnormalities, suggesting that methylation plays a role in early development (Branno et al., 1993; Zagris and Podimata, 1994; Cummings, 1994).

The zebrafish embryo offers a useful system for investigating epigenetic mechanisms that control development in vertebrates. The embryos can be readily obtained, are optically clear, and permit easy visualization of developmental events. The available genetic and molecular tools permit detailed molecular studies to be undertaken. Because the embryos develop externally, they can be treated with metabolic inhibitors added to their tank water, and the effects of the treatment can be evaluated without potentially complicating effects on the mother such as those encountered with mammalian models. In addition, the study of DNA methylation in mammals is complicated by the phenomenon of genomic imprinting, which appears to rely at least in part on DNA methylation for the control of parental allele expression (John and Surani, 1996). Although earlier studies indicate some possible parental origin effects on transgene expression (Martin and McGowan, 1995a), the zebrafish embryo does not appear to be highly subject to the genomic imprinting effects (Corley-Smith et al., 1996), thus making it a simpler model system with which to investigate the effects of DNA methylation. Except for studies of the relationship between DNA methylation and zebrafish transgene expression (or the lack of) (Gibbs et al., 1994; Martin and McGowan, 1995b), virtually nothing is known about DNA methylation in the zebrafish or its role in development of teleosts and other nonmammalian vertebrates.

To investigate the possible role of DNA methylation in normal zebrafish development, we characterized the temporal and spatial patterns of MTase expression during embryogenesis and evaluated the phenotypic consequences of treatment with methylation inhibitors. We observe striking phenotypic abnormalities in notochord, somite, and muscle development in the treated embryos. These data indicate a likely role for DNA methylation in supporting normal gastrulation and axial development in the zebrafish embryo.

MATERIALS AND METHODS

Animals

Embryos were obtained from zebrafish purchased from a local supplier. Fish were maintained using standard methods (Westerfield, 1995). Embryos were maintained at 28.5°C and staged according to hours (h) postfertilization (Kimmel et al., 1995).

5-Azacytidine, 5-Aza-2-deoxycytidine, and 6-Azacytidine Treatment

5-Azacytidine, 5-aza-2-deoxycytidine, and 6-azacytidine (Sigma) were dissolved in distilled water. Embryos were treated with a final concentration of 50 or 75 µM in embryo medium (Westerfield, 1995). Experiments were performed on embryos with their chorions intact or removed with pronase (Westerfield, 1995) with similar efficiencies. After the treatment period, embryos were washed extensively in embryo medium.

The optimal concentrations of cytidine analogues were determined empirically over a wide range of concentrations by producing a dose-response curve, in which embryos received the drug between 0 and 24 h after fertilization. The embryos were examined at several time points during treatment. A 100 µM concentration of 5-azaC produced overall mortality rates of 65%, whereas 25 µM 5-azaC resulted in less than 10% embryos with abnormal phenotypes. The dose-response curve for 5-aza-2-deoxycytidine resembled that for 5-azaC with lower mortality rates at higher concentrations. Concentrations of 6-azaC ranging from 5 to 100 µM had no apparent effect on the embryos, at 0-24 h.

Northern Analysis and in Situ Hybridization

Total RNA was extracted from zebrafish embryos using Trizol reagent (Life Technologies). Twenty micrograms of total RNA from each sample was denatured and subjected to electrophoresis in a formaldehyde gel according to Sambrook et al. (1989). Northern blots were prepared and hybridized with a 2.4-kb zebrafish MTase cDNA fragment labeled with 32P using the random hexamer labeling procedure. After hybridization, northern blots were subsequently stripped and re-probed with the zebrafish Max gene which provides a suitable control for equal loading and RNA integrity (Schreiber-Agus et al., 1994).
Embryo fixation and in situ hybridization were performed as described (Akimenko et al., 1994). The RNA probes were made from the following cDNA fragments: zebrafish MTase, 2.4 kb; α-tropomyosin, 1.1 kb (Weinberg et al., 1996); axial, 1.75 kb (Strähle et al., 1993); floating head, 0.7 kb (Tabot et al., 1995); no tail, 2.2 kb (Halpern et al., 1993; eng 2, 2.6 kb (Ekker et al., 1992a; knx20, 1.9 kb (Oxtoby and Jowett, 1993); FGF4, 1.4 kb (Thisse et al., 1995); and shh/vhh, 1.44 kb (Krauss et al., 1993).

DNA Isolation, End-Labeling, and Southern Analysis

DNA from 24-h untreated control and 5-azaC-treated embryos was isolated according to Sapienza et al. (1987). Mature sperm was collected from 12 male fish. DNA was prepared from the offspring of random breedings containing approximately 1000 blastula-stage embryos, 500 gastrula-stage embryos (6 h), and similar numbers of embryos collected at 16 or 24 h. Quality and concentrations of the DNA prior to digestion with endonucleases were verified by agarose gel electrophoresis followed by ethidium bromide staining. DNA was digested 3 h to overnight with HpaII or MspI. Complete digestion was tested by the addition of control plasmid DNA (pBluescript KS+) to an aliquot of the experimental digest. Digest was considered complete when the internal control digest gave a pattern identical to that obtained with the control plasmid alone. Southern blots containing 5 μg of HpaII- and MspI-digested DNA from 24-h treated and untreated embryos were prepared and hybridized to a probe corresponding to the zebrafish interspersed repetitive sequence DANA/mermaid (Izsvák et al., 1996; Shimoda et al., 1996a,b) and labeled with 32P using the random hexamer procedure. Blots were analyzed using a Bio-Rad GS-525 Molecular Image System and Image (version 1.61) digital image processing software written by Wayne Rasband (U.S. National Institutes of Health, Bethesda, MD). Visual examination of the gels stained with ethidium bromide prior to transfer showed relative degrees of DNA methylation can then be compared between samples by obtaining a ratio between the lane density of the HpaII- and MspI-digested DNAs.

RESULTS

MTase Expression in Zebrafish

For DNA methylation to contribute to the epigenetic changes that occur during embryogenesis, the DNA methyltransferase, a key enzyme in DNA methylation, must be expressed during development and may exhibit developmentally regulated changes in abundance or activity. A 2.4-kb partial cDNA coding putatively for the zebrafish DNA methyltransferase (MTase) was isolated during an EST screening project (GenBank Accession No. AA566662; Gong et al., 1997). Sequence of this cDNA over its entire length indicates that it encodes an enzyme highly similar to DNA methyltransferases from vertebrates (Fig. 1), other eukaryotes, and prokaryotes (not shown). For example, the overall amino acid sequence identity with the Xenopus MTase is 86%, with the mouse MTase, 80%. Several motifs are almost completely identical to their counterparts in other vertebrate MTase sequences (Fig. 1).

We performed Northern blot analysis on RNAs collected from blastula-stage (4 h), gastrula-stage (6 h), somite-stage (16 h), and tail-stage (24 h) embryos using the MTase cDNA as a probe. A transcript of approximately 5.0 kb is observed in all samples. The MTase mRNA abundance is greatest in blastocyst-stage embryos and then decreases dramatically during development to the gastrula stage (Fig. 2). MTase mRNA levels increase slightly in abundance compared to the gastrula stage during further development to the somite and tail stages.

Whole mount in situ hybridization using an antisense MTase riboprobe revealed that the MTase mRNA is abundantly and ubiquitously expressed in embryos from the time of fertilization (Fig. 3A and data not shown), demonstrating that MTase transcripts are of maternal origin, since embryonic transcription in zebrafish, starts at around the 1000-cell stage. In situ hybridization produces a similarly strong and ubiquitous signal in 3-h blastula-stage embryos (Fig. 3B) and in 6-h gastrula-stage embryos (Fig. 3C), but the latter require a longer reaction time to obtain signals comparable to those obtained with earlier stages, consistent with lower apparent MTase mRNA abundance observed by Northern hybridization (Fig. 2). The hybridization signals seen at 16 h are strongest in the brain, neural tube, and newly formed anterior somites (Figs. 3D and 3E). In tail-stage embryos (24 h), MTase mRNA expression is strongest in the eyes, central nervous system, and the somites of the tail bud (Figs. 3F and 3G). The shift of hybridization signal from anterior to posterior somites indicates that the MTase mRNA was expressed in a developmentally regulated manner, with the strongest expression in newly formed somites, followed by downregulation as the somites further develop. At high magnification, it is apparent that within each somite, MTase mRNA expression was strongest in a vertical row of cells at the anterior boundary of each somite (Fig. 3G).

Dynamics of DNA Methylation during Early Zebrafish Development

It is also expected that if DNA methylation contributes to the control of normal embryogenesis, then developmentally regulated changes in DNA methylation should occur. To learn whether such changes occur in the zebrafish embryo, DNA samples from sperm and pooled embryos of different stages were digested with the restriction enzyme HpaII or its isoschizomer MspI, both of which cut DNA at the sequence CCGG. MspI cleaves this sequence whether it is methylated or unmethylated, whereas HpaII only cleaves this sequence in the absence of methylation (Waalwijk and Flavell, 1978). We hybridized Southern blots with the zebrafish repetitive sequence DANA/mermaid (Shimoda et al., 1996a,b), which is interspersed and represents 5–10% of the zebrafish genome. By digesting samples with both enzymes, the relative degree of DNA methylation can then be compared between samples by obtaining a ratio between the lane density of the HpaII- and MspI-digested DNAs.
FIG. 1. Alignment of deduced amino acid sequences of MTases from zebrafish and other vertebrates. The multiple alignment was performed with Clustal V (Higgins et al., 1991). The symbols "*" and "." indicate amino acid identity and similarity, respectively, in all five sequences. Conserved domains such as the KG repeat and motifs I, II, IV, VI, VII, VIII, IX, and X are indicated. Only the protein region corresponding to the sequence coded by the zebrafish cDNA is indicated. Sequence accession numbers for the 2.4-kb MTase sequence from zebrafish (AF097875), chicken (D43920), human (X63692), mouse (X14805), and Xenopus (D78638).

<table>
<thead>
<tr>
<th>Species</th>
<th>Sequence</th>
<th>Zebrafish</th>
<th>Human</th>
<th>Mouse</th>
<th>Chicken</th>
<th>Xenopus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zebrafish</td>
<td>AGQQVJAQTRRAIILAAAGPHELFLPELFYTRAPACLSGWVDEDKSY</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>AGQQVJAQTRRAIILAAAGPHELFLPELFYTRAPACLSGWVDEDKSY</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>AGQQVJAQTRRAIILAAAGPHELFLPELFYTRAPACLSGWVDEDKSY</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chicken</td>
<td>AGQQVJAQTRRAIILAAAGPHELFLPELFYTRAPACLSGWVDEDKSY</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xenopus</td>
<td>AGQQVJAQTRRAIILAAAGPHELFLPELFYTRAPACLSGWVDEDKSY</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

Copyright © 1999 by Academic Press. All rights of reproduction in any form reserved.
This ratio is indicative of the degree to which the test samples approach complete hypomethylation.

Mature sperm DNA is hypermethylated relative to all other developmental stages tested (Fig. 4, lane 1). Oocyte DNA was not examined due to the difficulty in obtaining a sufficient number of oocytes. DNA methylation in the embryonic genome undergoes a large decrease during development, compared to sperm DNA, to its lowest level at 4 h postfertilization and remains low until at least 6 h (Fig. 4, lanes 1–3). In somite- and tail-stage embryos, the genome becomes once again heavily methylated (Fig. 4, lanes 4 and 5).

Experimentally Induced Hypomethylation with Cytidine Analogs Perturbs Embryonic Development

If DNA methylation has a significant role in normal development of the zebrafish, then we expect that inactivation of MTase during specific stages of development would result in dramatic perturbations in normal morphogenesis. We treated developing zebrafish embryos with 50 or 75 µM 5-azaC starting immediately after fertilization. Approximately 30% of embryos treated with such doses of 5-azaC show a shortened trunk and tail and loss or abnormal development of somites when the drug was administered from 0 to 24 h postfertilization and the embryos were examined at the end of the treatment. This is the most often observed phenotype, although head development is also perturbed in some treated embryos. At 48 h, these embryos display a beating heart, normal head and eyes, loss of tail or short tail, block-shaped somites, and an enlarged pericardial cavity (Figs. 5A and 5B). The trunk muscles of these abnormal embryos show distinct muscle fibers. However, the fibers are poorly organized and the somites failed to form chevron shaped myotomes (Figs. 5C and 5D). Mortality levels of 10 and 30%, above untreated controls, were observed when treating with concentrations of 5-azaC of 50 and 75 µM, respectively. A number of studies have used the cytidine analog 6-azacytidine (6-azaC) as a control for cytotoxic effects (Jones and Taylor, 1980; Doerksen and Trasler, 1996). This molecule is identical to 5-azaC except that the azide group is on position 6 of the cytidine ring. It does not affect DNA methylation. We treated zebrafish embryos with concentrations of 6-azaC ranging from 5 to 100 µM and did not observe any effects on development. We also treated embryos with another cytidine analog, 5-aza-2-deoxycytidine (5-azadC), which produces DNA hypomethylation similar to 5-azaC, but is not known to produce any general cytotoxic effects. Treatments with this drug produced identical results to those observed in embryos treated with 5-azaC. This suggests that the abnormal development of 5-azaC-treated and 5-azadC-treated embryos is the consequence of experimentally induced DNA hypomethylation.

To determine if 5-azaC is acting at a specific stage in development to produce the abnormal axial phenotype, we conducted a time-course experiment on developing embryos. Figure 6 shows the developmental periods for which embryos were treated with 5-azaC and the resulting percentage of embryos at 24 h that exhibit a “short tail” phenotype. More than 150 embryos were used for each treatment. Few embryos show any phenotype when 5-azaC is administered for the first 2 h after fertilization and then removed. Embryos that received 5-azaC between 2 and 3 h are almost as affected as those that received the drug from fertilization to 24 h. However, very few embryos receiving 5-azaC starting at 6 h show a phenotype. These results suggest that the period during which embryos are sensitive to 5-azaC is centered around 2–3 h after fertilization, a time that corresponds to the stages of high MTase mRNA expression (Figs. 2 and 3), just prior to midblastula transition.

Southern blot analysis, like that used to determine DNA methylation during development, was consistent with reduced DNA methylation in 5-azaC-treated embryos compared to 6-azaC and untreated control embryos (Fig. 7). Results consistent with the above Southern blot analysis were obtained by visual inspection of the ethidium bromide-stained gel prior to transfer or using the less sensitive end-labeling procedure (Monk et al., 1987; data not shown).

Abnormal Expression of Axial Markers in 5-AzaC-Treated Gastrula

The results of the time-course experiment indicate that 5-azaC must be administered during blastula and early gastrula to produce the short-tail phenotype. We have
looked at the expression of no tail, floating head, and axial, genes which are both expressed in and thought to be involved in the development of the axial mesoderm (Halp-ern et al., 1993; Talbot et al., 1995; Strähle et al., 1993). In untreated embryos, at 10 h postfertilization, cells in an elongated domain appearing on most of the length of the developing body axis express the three genes (Figs. 8A, 8C, and 8E), with a node of stronger expression occurring in the tail bud region. Patterns of expression of these genes in 5-azaC-treated embryos reflect a shortened and thickened

FIG. 3. Expression of DNA (cytosine-5) methyltransferase in zebrafish embryos. Whole mount in situ hybridization of (A) 8-cell-stage, (B) blastula-stage (3 h), (C) 6 h gastrula-stage, (D, E) 13-h somite-stage and (F, G) 24-h zebrafish embryos with a zebrafish MTase antisense RNA probe. Arrows in E and G indicate the anterior and posterior differentiating somites, respectively. Scale bar: A–D, 135 μm; E, 95 μm; F, 200 μm; G, 50 μm.
axial mesoderm (Figs. 8B, 8D, and 8F). The region of strong expression in the tail bud is absent in 5-azaC-treated embryos. The intensity of the expression signals in individual cells does not appear to be affected by 5-azaC. Twenty to 30% of 10 h 5-azaC-treated embryos display these abnormal expression patterns, a percentage similar to

FIG. 4. Global changes in DNA methylation during early zebrafish development. DNA collected from sperm, blastula stage (4 h), gastrula-stage embryos (6 h), somite-stage embryos (16 h), and 24-h embryos were digested with HpaII or MspI, and hybridized to the zebrafish interspersed repetitive sequence DANA/mermaid. Densitometric scans were performed in regions of the gel corresponding to low- and mid-molecular-weight DNA restriction fragments (region between left arrows). Mean density values (indicated here for this particular gel) were used to produce a comparative density ratio between the corresponding HpaII and MspI lanes. A high ratio indicates low levels of DNA methylation (i.e., high similarity between the densities of HpaII and MspI lanes) and low ratio indicates higher levels of DNA methylation. Ratios are indicated as the average of three independent gels ± SD.

<table>
<thead>
<tr>
<th>Lane</th>
<th>HpaII</th>
<th>MspI</th>
<th>Ratio</th>
<th>Mean Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>27</td>
<td>108</td>
<td>0.25</td>
<td>0.32±0.05</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>74</td>
<td>0.41</td>
<td>0.47±0.06</td>
</tr>
<tr>
<td>3</td>
<td>44</td>
<td>97</td>
<td>0.45</td>
<td>0.51±0.05</td>
</tr>
<tr>
<td>4</td>
<td>30</td>
<td>104</td>
<td>0.29</td>
<td>0.35±0.06</td>
</tr>
<tr>
<td>5</td>
<td>24</td>
<td>102</td>
<td>0.24</td>
<td>0.29±0.07</td>
</tr>
</tbody>
</table>

*mean±SD ratio calculated from 3 gels
the percentage of treated embryos showing the short-tail phenotype after 24 h of development.

Abnormal Notochord and Muscle Development in 5-AzaC-Treated Embryos

The phenotype caused by 5-azaC treatment resembles in some aspects that seen in the zebrafish no tail and floating head mutants which are known to completely lack a differentiated notochord (Halpern et al., 1993; Talbot et al., 1995). The resemblance of the 5-azaC- and 5-azadC-treated embryos with these zebrafish notochord mutants prompted us to investigate the development of the notochord in these embryos.

The region underlying the neural keel of 5-azaC-treated embryos, where the notochord normally resides, appears to be filled with undifferentiated mesoderm and cells that express α-tropomyosin, a marker of terminally differentiated muscle (Figs. 5C–5H). While muscle fibers expressing alpha-tropomyosin are present in 5-azaC-treated embryos, the muscle does not become properly organized into chevron-shaped myotomes (not shown). Transverse sections of 24-h zebrafish show a conspicuous notochord underlying the neural keel of wild-type embryos (Figs. 5E and 5F) and the absence of a differentiated notochord in 5-azaC-treated embryos (Figs. 5G and 5H). Notochord is observed in some embryos displaying the short tail phenotype; however, in these cases, a normal vacuolated notochord is primarily restricted to anterior portions of the trunk.

To better define the phenotype caused by 5-azaC treatments, we have examined the expression of no tail (Schulte-Merker et al., 1994) and eng2 (Ekker et al., 1992), genes which are at least in part responsible for differentiation of the notochord and muscle patterning, respectively (Halpern et al., 1993). By 19 h, no tail expression is limited to the differentiated notochord and the caudal mesoderm of the tail bud (Fig. 9A). 5AzaC phenotypic embryos show large gaps (Fig. 9B) in no tail expression in the notochord or near complete loss of no tail-expressing cells (Fig. 9C). Since the external appearance of 24 h 5-azaC-treated embryos most closely resembles that of 19-h untreated embryos, we present a comparison of these stages; however, consistent results are observed when comparing 5-azaC-treated with 24-h untreated embryos. Mosaic analysis using wild-type and no tail mutant embryos has demonstrated a requirement for no tail signaling to induce eng protein expression in

---

**FIG. 5.** Zebrafish embryos treated with 5-azacytidine lack tails and a differentiated notochord. (A) Wild-type embryos at 48 h of development have an elongated tail and possess a fully differentiated notochord in the trunk and tail. (B) Approximately 30% of embryos treated with 50 or 75 μM 5-azacytidine show reductions in the elongation of the tail, and loss or abnormal development of posterior somites. Structures anterior to the trunk appear to develop normally. (C, D) Transcripts of the muscle-specific α-tropomyosin gene are restricted to lateral bands along the body axis in 24-h wild-type embryos (dorsal view, C) but are also detected in the tissues that underlie the neural tube in 5-azaC-treated embryos (arrow, D). Transverse sections of (E, F) 24-h control and (G, H) 5-azaC-treated embryo hybridized with the α-tropomyosin probe. Control embryos show a large vacuolated notochord (n) directly beneath the neural keel (nk) and well-developed lateral somites (s), while sibling 5-azaC-treated embryos completely lack tissues resembling a notochord (arrow). (E and G, transverse sections posterior to hindbrain; F and H, transverse sections within posterior trunk/tail). Scale bar: A, 300 μm; B, 250 μm; C, 280 μm; D, 150 μm; E–H, 110 μm.

---

**FIG. 6.** Time course of the effects of 5-azaC on the development of axial structures. Embryos were treated with 5-azaC for the periods represented by the solid lines. The percentage of embryos showing a “short-tail” phenotype is indicated.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>% Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-24 h</td>
<td>30%</td>
</tr>
<tr>
<td>1-2.5 h</td>
<td>15%</td>
</tr>
<tr>
<td>2-3 h</td>
<td>21%</td>
</tr>
<tr>
<td>3-6 h</td>
<td>11%</td>
</tr>
<tr>
<td>3-24 h</td>
<td>14%</td>
</tr>
<tr>
<td>6-24 h</td>
<td>2%</td>
</tr>
</tbody>
</table>
the muscle pioneer cells of the developing somites (Halpern et al., 1993). In 5-azaC-treated embryos, expression of eng2 transcripts in the muscle pioneer cells is almost completely absent along the trunk and tail (Figs. 9E and 9F) compared to that of controls (Fig. 9D). While loss of eng2 expression is observed in 5-azaC-treated embryos, these same embryos showed normal expression of eng2 in the mid/hindbrain boundary. Loss of eng expression in muscle pioneer cells correlates with failure in proper myotome formation. Since we observed virtually no eng2
expression in 5-azaC-treated embryos, we wished to determine if the little expression we did observe, outside the midbrain/hindbrain boundary, is located in regions which possess no tail-expressing notochord cells. We performed double in situ hybridization labeling on embryos with both no tail and eng2 antisense probes. Along with control embryos, 5-azaC-treated embryos which showed fragments of no tail-expressing notochord cells were sectioned to correlate both no tail in the notochord and eng2 expression in muscle pioneers. In control embryos, no tail and eng2 transcripts are simultaneously expressed in the notochord and muscle pioneers, respectively (Fig. 9G). However, we do not see any eng2 expression in muscle pioneer cells even in sections of 5-azaC-treated embryos that have no tail-expressing notochord cells (Fig. 9H).

Development of the brain and neural keel does not appear to be greatly affected in 5-azaC-treated embryos. To further examine the effects of 5-azaC treatment on CNS development, we performed in situ hybridization using probes for a number of genes expressed in this tissue. The expression of genes such as krxd20 in the 3rd and 5th rhombomeres of the brain (Oxtoby and Jowett, 1993) and Z-FGFR4 expressed in the telencephalon, diencephalon, and the hindbrain (also expressed in the tail bud; Thisse et al., 1995) are normal (data not shown). The expression patterns of eng2 in the midbrain/hindbrain boundary (Ekker et al., 1992a) (see Fig. 9) and msxC in the neural keel (Ekker et al., 1992b; data not shown) are also normal. The floorplate in 5-azaC-treated embryos is present (as observed by in situ hybridization with a shh/vhh-1 probe [Krauss et al., 1993]); however, a number of abnormalities in the structure of the floorplate are observed such as discontinuities, kinking, and terminal forking (not shown). This is consistent with our understanding of a role for notochord in inducing floorplate. The phenotype of the embryos treated with 5-azaC suggests that

---

**FIG. 8.** Zebrafish embryos treated with 5-azacytidine have a shortened and thickened axial mesoderm. Expression of the genes no tail (A, B), floating head (C, D), and axial (E, F) occurs in the axial mesoderm of 10-h embryos. (A, C, E) Controls, (B, D, F) embryos treated with 50 μM 5-azaC from fertilization. Scale bar: A, B, E, and F, 500 μm; C and D, 580 μm.
this drug's effect is to perturb the development of the axial mesoderm.

**DISCUSSION**

**Dynamics of DNA Methylation and Methyltransferase Expression in Zebrafish Development**

The zebrafish MTase gene is developmentally regulated and expressed in a tissue-specific manner. Combined with stage-specific changes in DNA methylation, our data indicate that DNA methylation fulfills at least many of the requirements to allow us to suggest that it plays a role in the development of the zebrafish. MTase mRNA is maternally deposited and levels decrease abruptly after blastula. This decrease may be attributable to the degradation of the maternal transcripts. The exact time of onset of zygotic MTase expression during zebrafish embryogenesis is not known. MTase protein distribution in mice and observations of the MTase “knockout” mice suggest that active maternally deposited stores of MTase can persist in the embryo until at least 9.5 days postfertilization (Carlson et al., 1992; Li et al., 1992). At present, we know nothing about the stability of MTase in the zebrafish due to the absence of an antibody specific for zebrafish MTase or that will cross-react with zebrafish MTase. If it is as long-lived as in the mouse, MTase present in blastula-stage zebrafish embryos could persist throughout the entire embryonic development period which occurs over a couple of days. The tissue-specific expression of MTase in the zebrafish at later stages of development would seem to argue against a long-lived MTase protein.

During and after somitogenesis, we see highest expression of MTase in the neural tissues and within developing somites. The observation of high MTase expression in neural tissues has been reported in mice (Goto et al., 1994; Trasler et al., 1996). In the adult brain, high levels of MTase activity are in part thought to maintain genes in a non-expressed state by methylating genes no longer required for that cell lineage and for restricting expression of some genes within specific cell types of the somite. Patterns of myoD expression at 12 h postfertilization are mildly affected in 5-azaC-treated embryos: the longitudinal rows of expressing cells appear normal but the shape of the bands of cells that project laterally (Weinberg et al., 1996) is abnormal (data not shown). Surprisingly, we did not see a strong myoD expression in the midline where the notochord should form except for a few expressing cells (not shown), as we would have anticipated from the altered expression of muscle markers at later stages.

High MTase activity during blastula and gastrula stages has been observed in sea urchin, chick, and mouse. An interesting aspect of the relatively high MTase expression observed in blastula and gastrula of at least mouse and zebrafish is that the expression does not translate immediately into a relative increase in genome DNA methylation. Following fertilization, the diploid genome of the early zebrafish embryo undergoes a pronounced overall demethylation until approximately blastula stage with stages later than gastrula showing global methylation levels similar to the high methylation observed in sperm (Fig. 4). Monk et al. (1987) observed similar genomewide demethylation in early mouse embryos, even in the presence of large amounts of the MTase enzyme (Howett and Reik, 1991; Monk et al., 1991). This paradox may result from the absence, in pregastrula, of DNA methylation.
regulatory factors that are required for methyltransferase activity and the localization of methyltransferase to the peripheral cytoplasm of early embryonic cells (Carlson et al., 1992). The increase in DNA methylation following gastrulation occurs during a time of rapid cellular differentiation. Monk (1986, 1995) proposed that methylation events occur as part of an epigenetic program to stabilize or permanently repress genes particularly those located on the inactive X chromosome (mammals) no longer required for a specific cell lineage. The increases in DNA methylation following gastrula in zebrafish are similar to the pronounced changes observed during mouse development. Thus, DNA methylation may play a similar role in cementing cell differentiation in the development of non-mammalian vertebrates.

Similar changes in DNA methylation have been observed at various repetitive sequences in the mouse (reviewed by Yoder et al., 1997). In the present study we have used one such repetitive sequence, DANA/mermaid, as hybridization probe in the blots used to measure DNA methylation (Figs. 4 and 7). The design of this experiment is such that the hybridization signal provides an estimate of the overall DNA methylation, which was confirmed by the endlabelling procedure (not shown), and not of the DANA/mermaid sequence itself. The methylation status of the DANA/mermaid sequence at various stages of development remains to be determined.

Impaired DNA Methylation Affects Notochord and Somite Development

Treatments of zebrafish embryos with 5-azaC result in DNA hypomethylation in vivo and in abnormal development. Treated embryos lack a notochord, in whole or in part, and the mesoderm occurring in the position of the notochord is differentiated muscle. The somites are poorly developed. The fact that few other tissues appeared to be affected in these embryos suggests that methylation is specifically important in the differentiation of the axial mesoderm and, directly or indirectly, of the paraxial mesoderm.

Treatments of zebrafish embryos with 5-azaC at different stages of development indicate that the effective interval during which the drug must be administered to produce the axial phenotype occurs during cleavage stage, extending into a time period which includes the blastula and the very early gastrula stage. Expression of MTase is higher at these stages than at later times, although the genome is, overall, less methylated. We do not have, at this time, an explanation for this paradox. We suggest that changes in MTase structure or function are taking place around MBT and that perturbing these changes will have long-lasting effects. Possibilities include irreversible inhibition of the enzyme, impaired association with factors necessary for its entry into the nucleus, or conformational changes in the protein preventing either its entry into the nucleus or its function. The enzyme at later stages would be less sensitive to the effects of 5-azaC, perhaps because it is already nuclear and/or already active. It is clear that treatments initiated at 6 h have little effect, suggesting that the phenotype of the embryos is the result of events that took place around either 6 h or earlier. Such changes could include methylation of key genes (e.g., the first genes to be methylated). Therefore, treatments with 5-azaC may result in inappropriate gene expression shortly after midblastula transition, but before an overall increase in DNA methylation is detectable (Fig. 4). Such inappropriate expression has also been observed in MTase null mutants in the mouse (Li et al., 1993), and is evident in the altered protein patterns of 5-azaC-treated chick embryos (Zagris and Podlimateas, 1994).

Initially, we were concerned that the effects we observed could be the result of 5-azaC cytotoxicity. However, the action of 5-azaC appears to specifically affect the differentiation of the notochord and of structures such as the floor plate and muscle, whose development depends, in part, upon proper notochord function. As far as we could tell, initial differentiation of ectoderm and other mesodermal derivatives was unaffected. A correlation between affected tissues and parameters such as the rate of cell division could not be made. Time-course treatments have also shown that 5-azaC was only effective in producing the abnormal axial phenotype during a short period of time. Thus, the tissue specificity and temporal specificity of 5-azaC action are inconsistent with effects of a purely cytotoxic nature. Furthermore, treatments with 5-azaC, a drug known to have significantly lower cytotoxic effects, resulted in an identical abnormal phenotype in zebrafish embryos. Finally, treatments of zebrafish embryos with the cytidine analog 6-azaC resulted in no abnormal development at concentrations tested. This drug which does not affect DNA methylation, but whose structure is very similar to that of 5-azaC, has been used in a number of studies to control for 5-azaC cytotoxic effects (Jones and Taylor, 1980; Doerksen and Trasler, 1996). Therefore, the effects we observe on zebrafish development are likely due to abnormal DNA methylation.

The shorter and wider axial mesoderm in 5-azaC-treated embryos (Fig. 8) may be indicative of a disruption in axis elongation and/or improper intercalation of axial mesodermal cells during epiboly. The apparent overall decrease in expression of axial markers could be attributable to a lesser compaction of the cells in treated embryos compared to controls, suggesting a disruption in the normal intercalation movements of the axial mesoderm cells. Alternative explanations for the broadened patterns of axial marker expression could be ectopic expression of these three genes or a developmental delay. Similar changes in axial marker expression occur in floating head mutants (Halpern et al., 1995). Interestingly, axial structures are replaced by muscle in these mutants (Talbot et al., 1995) as we report here after
5-azaC treatment (Fig. 5). Our results are also consistent with studies in which 5-azaC-treated chick embryos displayed a shortened and thickened primitive streak (Zagris and Podimatas, 1994). However, these studies did not include gene expression analysis precluding a more direct comparison with our work.

Analysis of mouse T mutants and the zebrafish mutants no tail and floating head has revealed the requirement for these gene products for complete notochord differentiation (Halpern et al., 1993; Talbot et al., 1995). These mutations also demonstrate the requirement of a signal from the notochord for proper development of somites and of the floorplate. Zebrafish embryos treated with 5-azaC more closely resemble the phenotype of homozygous floating head mutant embryos. Both embryos show replacement of notochord by fusion of somites underneath the neural tube and disruption in the posterior floorplate. We cannot preclude that improper gene expression has induced apoptosis specifically in posterior floorplate. We cannot preclude that improper gene expression has induced apoptosis specifically in posterior floorplate.

ACKNOWLEDGMENTS

This paper is dedicated to the memory of William Steggles. We are particularly grateful to Keith Latham and Ross McGowan for helpful discussions. We thank Robert G. Haché, Keith Latham, Ross McGowan, and Sue Varmuza for critical reading of the manuscript. We thank Gary Hatch for technical assistance. We thank Tim Bestor for the suggestion to treat zebrafish embryos with MTase inhibitors. We thank Jacquetta Trasler for helpful comments and reviewing this work. We thank Z. Gong, M. Halpern, T. Jowett, S. Krauss, U. Strähle, W. Talbot, C. Thisse, E. S. Weinberg, and N. Schreiber-Agus for cDNA probes used in this study. C.C.M was supported by scholarships from the Natural Sciences and Engineering Research Council of Canada (NSERC), the Ontario Government, and the University of Toronto. This work was supported by grants from NSERC, CGAT, and MRC to M.E.

Note added in proof. Collas, P. (1998, Nucl. Acids Res. 26, 4454–4461) recently showed changes in the methylation of a plasmid DNA construct injected into zebrafish embryos that are consistent with the changes in methylation during development shown in the present report. Treatment of embryos with 5-azaC and sodium butyrate, as we previously showed (Martin and McGowan, 1995b), inhibits methylation of the plasmid and increases reporter expression.

REFERENCES


Received for publication June 4, 1998
Revised October 5, 1998
Accepted October 5, 1998